IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Axel ULLRICH et al. Examiner: BRISTOL, LYNN ANNE

Serial No.: 10/506,962 Group Art Unit: 1643

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Title: **USE OF EGFR TRANSACTIVATION INHIBITORS IN HUMAN CANCER**

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Professor Axel Ullrich, being duly warned, declare that

My CV is attached demonstrating my qualification to make this declaration.

I declare that I am a co-inventor of the present application and that I am employed by MAX-PLANCK-GESELLSCHAFT ZUR FOERDERUNG DER WISSENSCHAFTEN E.V. (the assignor of the present application) and that I have a financial interest under German law in whether a patent issues on the present application.

2. The following experiments were further conducted by me or under my supervision:

Goal: Identification of additional inhibitors of GPCR-induced EGFR transactivation.

Assay: The following assay is a standard technique for monitoring the effect of tyrosine phosphorylation

Methods:

The effect of anti-HB-EGF antibodies on LPA and Thrombin-induced stimulation of EGFR phosphorylation was examined in COS-7 cells. To this end, appropriately treated cells were lysed and tyrosine phosphorylation of the EGFR analyzed by colorimetric enzyme-linked immunosorbent assay (ELISA). Specifically, COS-7 cells were seeded in 6-well plates and cultured overnight at 37°C in 5% CO_2. Following starvation for 24 h, cells were pre-incubated for 1 h with polyclonal anti HB-EGF antibodies (20 μg/ml) or with the diphtheria toxin mutant CRM197 (10 μg/ml). Subsequently, cells were stimulated in triplicate with the GPCR agonists

LPA (10 μM) or thrombin (2U/ml) for 3 min at 37°C. Following stimulation, cells were lysed in 400 μl lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.5, 10 % glycerin, 5 mM EDTA pH 8.0, 1 % Triton-X 100, 20 rnM sodium pyrophosphate, 10 pg/ml aprotinin, 1 mM PMSF, 2 mM sodium orthovanadate, 100 mM NaF). After incubation on ice for 10 min, lysates (85 μl/well) were transferred to an ELISA micro-titer plate (Nunc Maxisorb), which had been coated overnight at 4°C with a monoclonal anti-EGFR antibody (rnAb 108.11 at 1.0 pg /ml; 100 μl/well), blocked with 150 μl/well blocking buffer (PBS, 0.5% BSA) and washed six times with washing buffer (PBS, 0.05% Tween 20) using an automated plate washer. Lysates were incubated on the antibody-coated plate overnight at 4°C with gentle agitation. Following washing (4X) with 100 μl washing buffer,100 μl of biotinylated anti-phosphotyrosine monoclonal antibody 4G10 (UBI) diluted to 0.2 μg/ml in dilution buffer (PBS, 0.5% BSA, 0.05% Tween 20, 5 mM EDTA) was added to each well and incubated for 2 h at room temperature (RT). The plate was washed and 100 μl HRP-conjugated streptavidin (UBI) diluted 1:40000 in dilution, buffer was added to each well and incubated for 30 min at RT. Free avidin conjugate was washed away (3X) and 100μl substrate solution (tetramethyi benzidine, TMB, Calbiochem) was added to each well and.incubated at RT with gently shaking in the dark. After 15 min, the reaction was stopped by addition of 100 μl/well 250 mM HCl and the absorbance at 450 nm was read with a reference wavelength of 550 nm using a microplate reader (Thermo Lab Systems).

Results are shown in Fig. 1

As depicted in Fig. 1, treatment with GPCR agonists LPA and Thrombin induced tyrosine phosphorylation of EGFR in COS-7 cells. In these experiments, the level of tyrosine phosphorylation was assayed using an ELISA assay (absorbance at 450 nM). However, treatment with anti-HB-EGF antibodies or the diphtheria toxin mutant CRM197 completely inhibited EGFR transactivation. For example, treatment with 20 μg/ml polyclonal anti-HB-EGF antibody inhibited LPA-induced activation of EGFR to basal levels. Similar results were obtained with respect to thrombin-induced activation of EGFR.

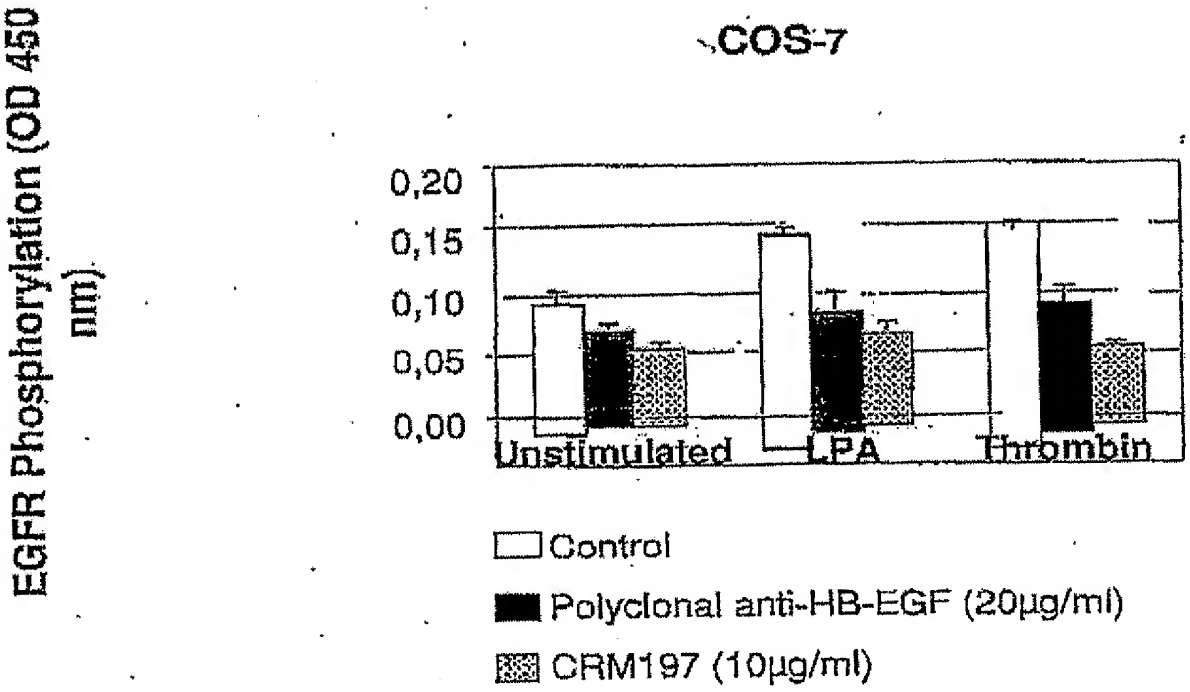

Fig. 1: Effect of GPCR agonists LPA and thrombin on tyrosine phosphorylation of the EGFR in COS-7 cells.

Conclusions: The aforementioned study demonstrates that inhibitors of the HB-EGF pathway, such as, for example, anti-HB-EGF antibodies, interfere strongly with GPCR-mediated EGFR activation and neutralize the activity of HB-EGF. Additionally, the results indicate that antibodies against HB-EGF are therapeutically useful in the treatment of hyper-proliferative diseases, particularly those that are associated with abnormal GPCR-induced receptor tyrosine kinase signaling.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

10/8/09
Date